The Myosin SH_2–50-Kilodalton Fragment Cross-Link: Location and Consequences†

Patrick Chaussepied,*,‡ Manuel F. Morales,‡ and Ridha Kassab§

Cardiovascular Research Institute, University of California, San Francisco, California 94143, and Centre de Recherches de Biochimie Macromoleculaire du CNRS, INSERM U.249, Université de Montpellier I, 34033 Montpellier Cedex, France

Received June 23, 1987; Revised Manuscript Received October 19, 1987

ABSTRACT: Some of us recently described a new interthiol cross-link which occurs in the skeletal myosin subfragment 1–MgADP complex between the reactive sulfhydryl group "SH_2" (Cys-697) and a thiol (named SH_x) of the 50-kilodalton (kDa) central domain of the heavy chain; this link leads to the entrapment of the nucleotide at the active site [Chaussepied, P., Mornet, D., & Kassab, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2037–2041]. In the present study, we identify SH_x as Cys-540 of the 50-kDa fragment. The portion of the heavy chain including this residue and also extending to Cys-522 that is cross-linkable to the "SH_1" thiol [Ue, K. (1987) *Biochemistry 26*, 1889–1894] is near the SH_2–SH_1 region. Furthermore, various spectral and enzymatic properties of the (Cys_{697}–Cys_{540})-*N*,*N*′-*p*-phenylenedimaleimide (pPDM)-cross-linked myosin chymotryptic subfragment 1 (S-1) were established and compared to those for the well-known (SH_1–SH_2)–pPDM-cross-linked S-1. The circular dichroism spectra of the new derivative were similar to those of native S-1 complexed to MgADP. At 15 mM ionic strength, (Cys_{697}–Cys_{540})–S-1 binds very strongly to unregulated actin ($K_a = 7 \times 10^6$ M^{-1}), and the actin binding is very weakly affected by ionic strength. Joining actin with the (Cys_{697}–Cys_{540})–S-1 heavy chain, using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide, produces different species than does joining unmodified S-1 with actin. In contrast to the SH_1–SH_2-cross-linked S-1 which is thought to resemble the M·ATP (M·ADP·P_i) state [Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4909–4913], the (Cys_{697}–Cys_{540})–S-1 conformation seems to simulate an M·ADP state.

Occasionally, it has been possible to join covalently two points of the skeletal chymotryptic myosin subfragment 1 (S-1)[1] with a small bifunctional molecule; thus, thiols "SH_1" and "SH_2" have been joined with *N*,*N*′-*p*-phenylenedimaleimide (Burke et al., 1976), and Cys-522 and SH_1 have been joined with dibromobimane (Mornet et al., 1985a; Ue, 1987). Such chemical cross-linkings are interesting because they establish physical proximities and because they sometimes "freeze" special conformations. Recently, some of us (Chaussepied et al., 1986a) discovered a third union, between SH_2 (which resides on the so-called 20-kDa domain) and a thiol whose sequence location was not determined but which was known to reside on the 50-kDa domain; we termed this a union of "SH_x" and SH_2. This new cross-linking extinguished Ca^{2+}-ATPase and resulted in "trapping" of Mg nucleotide, much as Wells and Yount (1979, 1980) had found to follow SH_2–SH_1 cross-linking. Shortly after these findings, cross-linking experiments illustrating the nucleotide-dependent spatial relations between the 50-kDa region and the SH_1–SH_2 sites have been also described by others (Lu et al., 1986; Rajasekharan et al., 1987).

In the present paper, we first identify SH_x in the amino acid sequence of the 50-kDa heavy-chain segment, and then we report on several functional properties of the cross-linked S-1. Others (Chalovich et al., 1983) have pointed out that in some ways S-1 that has been SH_1–SH_2 cross-linked behaves like unmodified S-1 that is binding MgATP and/or ADP·P_i. Analogously, we find here that S-1 that has been SH_x–SH_2 cross-linked behaves in some regards like unmodified S-1 complexed to MgADP. Were these correlations later to be supported by independent structural evidence, we would be implying that with the two cross-links we are freezing two distinct important conformations of the myosin active site.

Materials and Methods

Materials. α-Chymotrypsin was from Worthington Biochemicals. *N*,*N*′-*p*-Phenylenedimaleimide was from Boehringer-Mannhein. Cyanogen bromide and EDC were from Sigma. [^{14}C]ADP was from New England Nuclear. $NaDodSO_4$, acrylamide, and all other gel electrophoresis chemicals were from Bio-Rad. *N*-Ethylmorpholine, dansyl chloride, dimethylformamide, and F-1700 micro-polyamide plates were from Pierce. All other chemicals were of the highest analytical grade.

Preparation of Proteins and S-1 Derivatives. Rabbit skeletal myosin was prepared as described by Offer et al. (1973). S-1 was obtained by digestion of myosin filaments

† The earlier part of this research was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale (CRE5-11850), the Direction des Relations et de la Coopération Internationales (AI-950321), and the Muscular Dystrophy Association of America; the later part was supported by USPHS Grant HL-16683, by NSF Grant INT8514204, and by AHA Grant CI-8. P.C. is a Career Investigator Fellow of the AHA, and M.F.M. is a Career Investigator of the AHA.

* Correspondence should be addressed to this author.

‡ University of California.

§ Université de Montpellier I.

[1] Abbreviations: S-1, myosin chymotryptic subfragment 1; LC1, alkali light chain 1; LC3, alkali light chain 3; SH_1, thiol residue 707; SH_2, thiol residue 697; M_r, molecular weight; CD, circular dichroism; pPDM, *N*,*N*′-*p*-phenylenedimaleimide; DNFB, 2,4-dinitrofluorobenzene; DNP-S-1, dinitrophenyl subfragment 1; IAA, iodoacetamide; NEM, *N*-ethylmaleimide; IAEDANS, *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine; DTE, dithioerythritol; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Mops, 4-morpholinepropanesulfonic acid; AEDANS, *N*-acetyl-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid.

0006-2960/88/0427-1778$01.50/0

with α-chymotrypsin according to Wagner and Weeds (1977) and purified as previously reported (Chaussepied et al., 1986b).

The split S-1 obtained by trypsin cleavage (trypsin:S-1 weight ratio 1:100, at 25 °C, for 30 min in 50 mM Tris-HCl, pH 8.0) was purified according to Mornet et al. (1980). Rabbit skeletal muscle actin was prepared as described by Eisenberg and Kielley (1974).

Specific Modifications of S-1 Thiols. The SH_1 group of S-1 was labeled with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (1,5-IAEDANS) according to Takashi et al. (1976). Using an absorption coefficient $A^{340nm} = 6.2 \times 10^3$ M^{-1} cm^{-1} for 1,5-IAEDANS (Hudson & Weber, 1973), we found that 1.1 mol of dye was incorporated per mole of S-1. The correlation between the loss of the K^+-ATPase activity and the fluorescence intensity distribution on the gel allowed us to quantify the labeling ratio as 0.95 in the heavy chain and 0.15 in the light chains.

The chemical modification of SH_1 by 2,4-dinitrofluorobenzene (DNFB) or by *N*-ethylmaleimide (NEM) or iodoacetamide (IAA) was carried out as in Reisler et al. (1974) and in Kunz et al. (1977), respectively. The degree of modification was evaluated in all cases by loss in K^+-ATPase activity and reached 0.9–0.95 for all reagents. The thiolysis procedure for dinitrophenylated S-1 previously reported (Chaussepied et al., 1986a) was improved as follows: 30 μM protein in 50 mM Hepes buffer, pH 8.0, was incubated in the presence of 10 mM DTE overnight at 4 °C. The thiolysis reaction was stopped by gel filtration through a Pharmacia PD10 column, and the K^+- and Ca^{2+}-ATPase activities showed the restoration to 85–90% of control S-1. We postulated that the thiolysis reaction has the same efficiency with the native S-1 and the cross-linked $(SH_2–SH_x)$–S-1.

Intramolecular Cross-Linking Reactions. The native or the SH_1-blocked S-1 enzyme (50 μM) in 50 mM Hepes buffer, pH 8.0, was reacted with a 1.3- or 2-fold excess of pPDM in the presence of 2.5 mM MgADP or Mg[^{14}C]ADP at 4 °C for 45 or 90 min, respectively (Wells & Yount, 1980; Chaussepied et al., 1986a). The fractions per mole of [^{14}C]ADP trapped were usually found to be 0.8–0.9 in $(SH_1–SH_2)$–S-1 and 0.75–0.85 in $(SH_2–SH_x)$–S-1. In each case, the excess of pPDM was removed by filtration through a PD10 column. The $(SH_1–SH_2)$–S-1 was further separated from un-cross-linked S-1 by sedimentation with actin as described by Chalovich et al. (1983). Unfortunately, the $(SH_2–SH_x)$–S-1 could not be separated from the un-cross-linked S-1 with the same procedure.

Protein Concentrations. The concentration of native S-1 was determined from its absorbance, assuming an absorption coefficient $A^{1\%}_{280nm}$ of 7.5 cm^{-1} (Wagner & Weeds, 1977). The modified or fragmented S-1 derivatives were evaluated according to Bradford (1976) with S-1 as a standard. The F-actin concentration was obtained by using $A^{1\%}_{278nm} = 11$ cm^{-1} (West et al., 1967).

Circular Dichroic Spectrum. CD spectra of S-1 derivatives were obtained as described previously (Murphy, 1974; Chaussepied et al., 1986b) on a computerized Jobin et Yvon Model Mark V autodichrograph.

Acto-S-1 Intermolecular Cross-Linking Reaction. The covalent EDC-induced cross-linking between F-actin and the S-1 derivatives was performed essentially as described by Mornet et al. (1981).

Actin Binding Measurements. The association of actin with the native or cross-linked S-1's was studied in the absence or presence of nucleotide at different ionic strengths by sedimentation in a Beckman airfuge (Chalovich & Eisenberg, 1982; Chaussepied et al., 1986b). The binding constant was measured in 10 mM Hepes, 5 mM NaCl, and 2 mM $MgCl_2$, mixing 5 μM S-1 with varying actin concentrations (0–100 μM). The fraction of unbound S-1 derivative in the supernatant was estimated from densitometric observations of bands on electrophoretograms, and the affinity constants were deduced from Scatchard plots.

S-1 ATPase Activities. The K^+/EDTA and Ca^{2+}-dependent ATPase activities of S-1 were measured as described by Mornet et al. (1979).

Cyanogen Bromide Cleavage and End-Group Analysis. AEDANS–S-1 and pPDM-cross-linked AEDANS–S-1 were cleaved by CNBr in 70% formic acid at 20 °C for 16–18 h using a 100-fold excess of CNBr over the methionine content (Elzinga & Collins, 1977). The purification of the CNBr peptides was accomplished as described by Ue (1987). The lyophilized peptide (3–5 mg) was dissolved in 8 M urea (5% $NaDodSO_4$, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue, and 50 mM Tris-HCl, pH 8.8) and electrophoresed on a preparative gel. The fluorescent bands were cut out, and the corresponding peptides were extracted from the gel, dialyzed against water, and lyophilized to dryness. Analysis of the N-terminal residues of each peptide was performed as described by Gray (1972) with modifications instituted by Ue (1987). The identification of the dansyl amino acids was made by using two-dimensional chromatography on polyamide layers with predansylated amino acid residues as standards.

Electrophoresis. The peptide species in various preparations were usually analyzed on polyacrylamide gradient gels (5–18%) as described by Laemmli (1970) and modified by Mornet et al. (1981). The preparative gel used for peptide purification was a 3-mm-thick, 10–18% gradient acrylamide gel.

The molecular weight standards used in the molecular weight determination were the fragments resulting from S-1 or proteolytic degradation of S-1 [known as 95, 50, 27, and 23 kDa (LC1) or 20 and 16.5 kDa (LC3)]. The densitometric scanning of the gels was carried out with a computerized Model CS 930 Shimadzu gel scanner.

RESULTS

Localization of pPDM Anchorage in the 50-kDa Fragment. We have previously shown that the reaction of pPDM with SH_1-blocked S-1 in the presence of nucleotide induces the formation of a link between the 20- and 50-kDa domains of the S-1 heavy chain (Chaussepied et al., 1986a). The loss of Ca^{2+}-ATPase activity correlated with the cross-link suggested SH_2 as the pPDM site in the 20-kDa fragment, but the pPDM site in the 50-kDa fragment remained unknown.

Besides SH_2, there are two other SH possibilities in the 20-kDa fragment (Cys-674 and Cys-792), and there are three SH_x possibilities in the 50-kDa domain (Cys-402, Cys-522, and Cys-540), if we postulate that under the conditions used pPDM predominantly reacts with thiols. To identify the thiols actually participating in the cross-link, we used the strategy recently developed by Ue (1987).

As detailed in Figure 1, extensive CNBr cleavage of the S-1 heavy chain yields seven peptides containing one or more thiols. Because CNBr treatment does not usually split pPDM, we expect that if we find the molecular weights and identify the N-terminal residue(s) of the CNBr peptides derived from $(SH_2–SH_x)$–S-1, we will discover the identity of SH_x. We first blocked the SH_1 residue with the fluorescent probe 1,5-IAEDANS; then we could detect all the SH_1-containing peptides that also contain the SH_2 group participating in the cross-link,

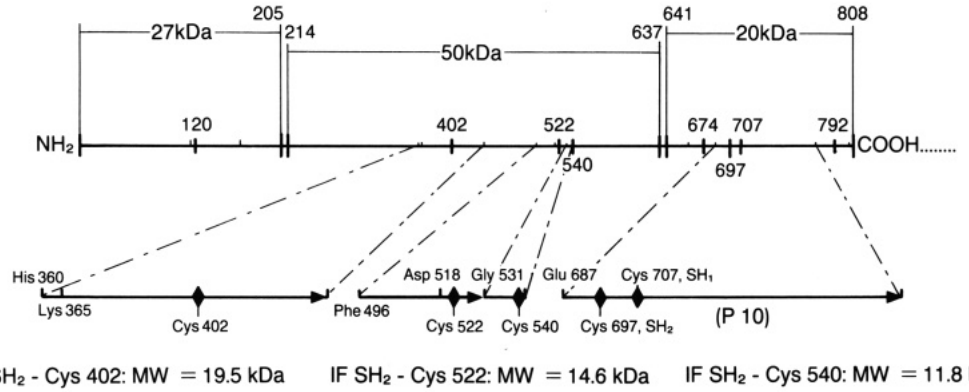

FIGURE 1: Predicted CNBr peptides involved in the pPDM-induced cross-link between SH_x of 50 kDa and Cys-697 of 20 kDa. Lys-356 and Asp-518 are N-terminal residues of two potential CNBr peptides induced by a heterogeneity at positions 355 and 517, respectively. The primary sequence information is from Ue (1987).

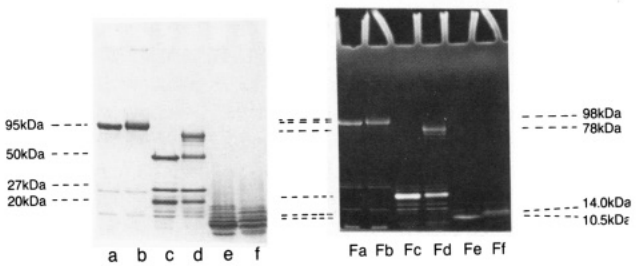

FIGURE 2: Representative $NaDodSO_4$–polyacrylamide gel showing tryptic digestion and CNBr cleavage products of un-cross-linked and (SH_2–SH_x)–pPDM-cross-linked S-1. AEDANS–SH_1–S-1 (a, Fa) or AEDANS–SH_1–(SH_2–SH_x)–pPDM–S-1 (b, Fb) was digested by trypsin (c, Fc and d, Fd, respectively) or cleaved by CNBr (e, Fe and f, Ff, respectively) as described under Materials and Methods. The gel was stained with Coomassie Blue (a–f) or viewed under ultraviolet light (Fa–Ff).

since SH_1 and SH_2 are not separable by CNBr cleavage. As shown in Figure 2 (lanes a and b), the result of the pPDM reaction on AEDANS–S-1 heavy chain is a species with a higher molecular weight than that of un-cross-linked S-1 (98K instead of 95K).

The tryptic degradation products of AEDANS–S-1 or of pPDM-cross-linked AEDANS–S-1 are shown in lanes c and d. As previously described, the cross-linked product of the 20- and 50-kDa fragments migrates with a molecular weight of 78K (Chaussepied et al., 1986a). In this particular experiment, the yield of the cross-linked product was 75%, and the cross-linking reaction generated a main 78-kDa band and a minor band migrating at 74 kDa (5% of the cross-linked species).

This pattern of results was usually obtained either in the presence of various polyphosphates, such as MgATP, MgADP, or $MgPP_i$, or in their absence, when using 3 molar excess of pPDM over S-1 (in an overnight reaction for the latter case). However, when the starting material was tryptically split S-1 (27 kDa–50 kDa–20 kDa) instead of the native S-1, the cross-linking reaction was influenced by the presence of Mg polyphosphate: in its presence, we still obtained a 78:74-kDa band ratio of 0.95:0.05; in its absence, the ratio was 0.60:0.40 throughout the entire course of the pPDM reaction (data not shown). Because these results apply only to tryptically split S-1, which exhibits some other structural and functional differences with native S-1 (Botts et al., 1982; Goodearl et al., 1985; Chaussepied et al., 1986b; Highsmith & Eden, 1987), we will henceforth consider the 78-kDa band as the only real product of pPDM reaction with SH_1-blocked S-1.

The distribution of the fluorescence in the gel electrophoretic pattern following CNBr cleavage (lanes e and f) shows two different bands, with molecular weights of 10.5K and 14.0K, on cleaving AEDANS–S-1 and pPDM-cross-linked AEDANS–S-1, respectively. The 10.5-kDa band is the "P10" CNBr peptide containing the fluorescent SH_1 and unmodified SH_2 residues (Elzinga & Collins, 1977).

To identify the composition of the 14-kDa band, we investigated its N-terminal residue in comparison to those of the P10 peptide. For this purpose, we carefully cut and eluted these two bands from the polyacrylamide gel and submitted them to end-group analysis (see Materials and Methods). In contrast to the 10.5-kDa peptide, which presented one dansylated Glu residue that is characteristic of the N-terminal residue of P10 peptide (Figure 1), the 14.0-kDa band exhibited mainly two dansylated residues, viz., Glu and Gly, indicating that this band consisted of two different peptides. One of them is the P10 peptide which contains fluorescence-labeled SH_1 and whose N-terminal group is a Glu; this result already indicates that SH_2 is involved in the 20-kDa–50-kDa link. The second peptide present in the 14.0-kDa band is certainly the Cys-540 containing CNBr peptide because of the following:

(i) The second N-terminal residue found is Gly. In the entire S-1 from rabbit skeletal muscle, there is only one CNBr peptide that begins with Gly-531, and this is the peptide that contains Cys-540. This fact eliminates the otherwise possible contamination of the 14.0-kDa band by a nonfluorescent peptide comigrating with the fluorescent cross-linked product (such a contaminant would have to have at least three uncleaved Met–amino acid linkages in order to have the same molecular weight).

(ii) The molecular weight of 14.0K is in good agreement with the molecular weight expected from adding this Cys-540-containing peptide to the P10 fragment: as shown in Figure 1, the real molecular weight should be 11.8K in comparison to 14.6K or 19.5K if the cross-linked cysteines were Cys-522 or Cys-402, respectively. As discussed by Ue (1987), who observed the same phenomenon, the interthiol cross-linked products generally exhibit a higher molecular weight than expected (e.g., 98K and 78K instead of 95K and 70K for the 50K + 20K cross-linked products), so the molecular weight of 14.0KDa can be more easily related to the 11.8-kDa band than to the higher bands of 14.6 or 19.5 kDa. We did not identify the cross-linking site when SH_1 was masked by smaller molecules such as IAA or NEM, but the fact that the 20-kDa–50-kDa cross-linked product obtained in all the cases has a molecular weight of 78K suggests that the nature of the SH_1-blocking agent does not affect this SH_2–Cys_{540}–pPDM bridge formation.

Circular Dichroic Spectral Characteristics of (SH_2–Cys_{540})–pPDM–S-1. Another way to study the ATP and ADP

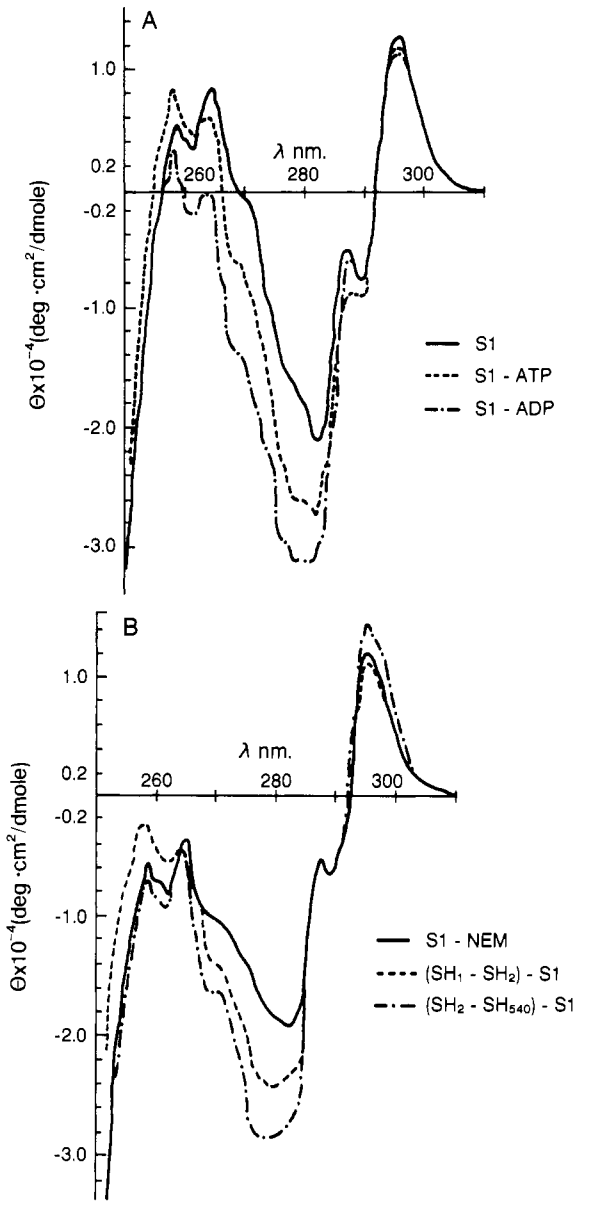

FIGURE 3: Comparative near-ultraviolet CD spectra of intramolecularly cross-linked S-1 derivatives. The spectra were measured with 45 μM protein in 0.2 M KCl and 50 mM Mops buffer, pH 7.0, at 20 °C. (A) The spectra of native S-1 were performed in the absence (—) and in the presence of 0.5 mM Mg^{2+}-ATP (---) or 0.5 mM Mg^{2+}-ADP (-·-). (b) The spectrum of NEM-SH_1-S-1 (—) is compared to those obtained with (SH_1-SH_2)-pPDM-S-1 (---) and (SH_2-Cys_{540})-pPDM-S-1 (-·-).

interaction with S-1 is to follow their unequal effects on the near-ultraviolet CD spectrum of S-1 between 250 and 310 nm (Murphy et al., 1974).

The spectrum of native S-1 (Figure 3A) is very similar to that already obtained for papain-S-1 (Murphy, 1974; Burke et al., 1976; Wu & Yang, 1976) or to that of chymotryptic S-1 (Chaussepied et al., 1986b). Note in this spectrum the effect of ATP and ADP binding on the region near 258 and 282 nm. The spectra recorded for the various cross-linked S-1's are in Figure 3B and are compared to NEM-SH_1-S-1. This derivative displays the same shape as the doubly modified NEM-SH_1, NEM-SH_2-S-1 (unpublished data; Burke et al., 1976). The overall shape of NEM-SH_1-S-1 is similar to that of unmodified S-1 except for the 260-nm region where the intensity is significantly decreased with modification. As previously described (Burke et al., 1976), the cross-link between SH_1 and SH_2 affects the CD spectrum of the NEM-modified S-1 in the same manner as MgATP affects the spectrum of the native S-1, namely, in the 260- and the 282-nm regions. On the other hand, the compared spectra of (SH_2-Cys_{540})-pPDM-S-1 and NEM-SH_1-S-1 differ from the native S-1 spectrum in much the same way as the MgADP-S-1 spectrum differs from the native S-1 spectrum. At 282 nm (260 nm could not be used because of NEM interference), we compared the ratio (θ) of the measured system to θ of the control. When the system was S-1 plus ATP or ADP and the control was S-1 alone, the ratios were 1.28 and 1.46, respectively. When the systems were (SH_1-SH_2)-S-1 and (SH_2-Cys_{540})-S-1 and the control was NEM-S-1, the ratios were 1.22 and 1.44, respectively. The spectrum of this new (SH_2-Cys_{540})-S-1 also shows a slight enhancement in the 300-nm region, usually related to the Trp side chain. This specific enhancement is not induced by ADP or ATP binding to the S-1 molecule (Figure 3A).

Table I: Comparative Constants for Binding of Actin to Various Nucleotide-S-1 Complexes or S-1 Derivatives[a]

K_a (M^{-1})	S-1	modified S-1[b]
alone	7.2×10^7[c]	6.5×10^7
ADP	6.2×10^6[c]	5.6×10^6
ATP	2.0×10^4[c]	1.8×10^4
(SH_1-SH_2)-S-1	3.2×10^4	
(SH_2-Cys_{540})-S-1		6.8×10^6

[a] The values were extracted from the Scatchard plots obtained from binding experiments performed as described under Materials and Methods. [b] The S-1 was first modified at the "SH_1" residue with DNFB and then submitted to the thiolysis step as described under Materials and Methods. In this particular experiment, the thiolysis allowed us to recover 89% of unmodified S-1. [c] Values from Chaussepied et al. (1986b), who used the same experimental conditions.

If we consider NEM-S-1 as the control with which we can compare the different pPDM-treated S-1's, it seems that the two pPDM-cross-linked S-1's exhibit two different CD spectra, suggesting that they are generated by two different conformations; if one is related to the M·ATP states, the structure of the new (SH_2-Cys_{540})-pPDM-S-1 could be related to the M·ADP states.

Actin Interaction Properties. The actin binding properties of (SH_2-Cys_{540})-S-1 were first studied by sedimentation. The values of the affinity constants obtained were compared to those of native S-1 in the presence or absence of nucleotide and to that of (SH_1-SH_2)-S-1 (Table I). The affinity of (SH_2-Cys_{540})-S-1 to unregulated actin is 6.8×10^6 M^{-1}; this value was obtained for an S-1 which suffered a reversed SH_1 modification; we note a decrease in affinity due to the cross-linking of 1 order of magnitude. This decrease is to be compared with the 2000-fold decrease induced by SH_1-SH_2 cross-linking (Table I). We then compare these values to the affinity constants of different S-1-nucleotide complexes for actin. Instead of resembling (SH_1-SH_2)-S-1 which exhibits a binding constant related to that of the S-1-ADP·P_i complex, as found by various authors (Chalovich et al., 1983; Katoh & Morita, 1984; Greene et al., 1986), we find that the new (SH_2-Cys_{540})-S-1 displays the greatest similarity to S-1 in complex with ADP ($K_a = 6.8 \times 10^6$ and 5.6×10^6 M^{-1}, respectively, as shown in Table I).

The different S-1-nucleotide complexes can also be characterized by the salt dependence of their actin interaction. Figure 4 shows the amount of actin-bound S-1 as a function of NaCl concentration: native S-1 or S-1-MgADP is not or only slightly affected up to 250 mM NaCl where, at worst, 70% of S-1 still interacts with actin. On the other hand, the actin-S-1-MgADP·P_i interaction is highly sensitive to salt since at 125 mM NaCl only 10% of S-1 interacts with actin.

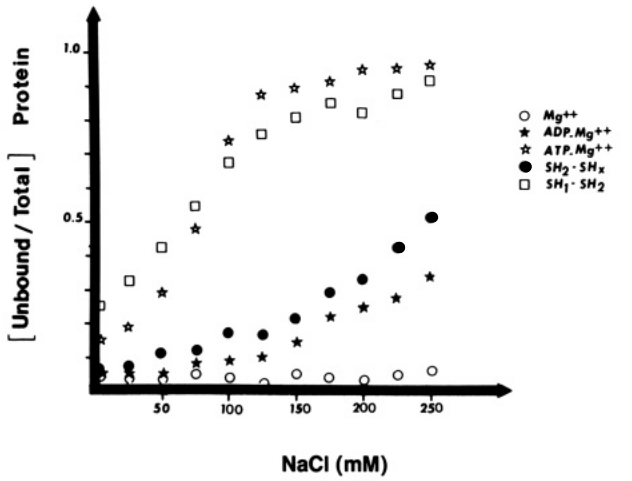

FIGURE 4: Influence of increasing NaCl concentrations on the association of actin with cross-linked S-1 derivatives. Cosedimentation experiments were performed as described under Materials and Methods, mixing 5 μM S-1 with a 4-fold excess of actin in each experiment. The NaCl effect was followed with native S-1 as control (containing 2.5 mM Mg^{2+}) in the absence (○) or in the presence of 2.5 mM ATP (☆) or 2.5 mM ADP (★). The salt dependence of the actin interaction with (SH_1–SH_2)–pPDM–S-1 (□) or (SH_2–Cys_{540})–pPDM–S-1 (●) was determined in the absence of free Mg^{2+} nucleotide.

When we study the NaCl effect on (SH_1–SH_2)–S-1 to actin binding, we find behavior very similar to that of the S-1–ADP·P_i (or S-1–ATP) complex. On the other hand, (SH_2–Cys_{540})–S-1 shows a salt dependence of its actin interaction that is very similar to that of the S-1–ADP complex, with 50% of the S-1 derivative still attached to actin at 250 mM NaCl.

In order to specify the acto-S-1 interface which exists in the (SH_2–Cys_{540})–S-1, we studied the EDC-induced intermolecular cross-linking of actin and the S-1 derivative. Figure 5A illustrates the results obtained with AEDANS–S-1 as control, and using (SH_2–Cys_{540})–AEDANS–S-1. While the cross-linked products obtained with SH_1-blocked S-1 display the well-known doublet band corresponding to 175- and 185-kDa peptides, actin cross-linking to pPDM-treated S-1 produces a single band, with a molecular weight of 150K. This 150-kDa band contains the fluorescence carried by the labeled SH_1 and also contains fluorescent actin when actin has previously been labeled by 1,5-IAEDANS and cross-linked to (SH_2–Cys_{540})–S-1 obtained after reversible SH_1 blocking (data not shown). The weaker bands at 175/185 kDa are assigned to the cross-linking of actin and residual unmodified S-1 (15–20% of the preparation).

We also carried out similar experiments with trypsin-split AEDANS–S-1 before and after pPDM modification (Figure 5B). The actin cross-linking with the 78-kDa band (originating from pPDM-induced cross-linking of the 20- and 50-kDa fragments) generated a product of M_r 140K identified as a complex of actin and the 78-kDa entity. The use of control tryptic S-1 resulted in the formation of fluorescent 20-kDa actin (62 kDa) and nonfluorescent 50-kDa actin (95 kDa). Note that the 65-kDa band, composed of 20- and 45-kDa fragments (the 45-kDa fragment derives from an overdigestion of the 50-kDa fragment), does not seem to cross-link with actin as shown by the lack of an acto-65-kDa band.

Discussion

The present results specify the location of the interthiol cross-link joining the 50- and 20-kDa domains of skeletal S-1 heavy chain (Chaussepied et al., 1986a). The loss of Ca^{2+}-ATPase activity accompanying formation of the intramolecular bridge suggested the involvement of thiol SH_2 (residue 697

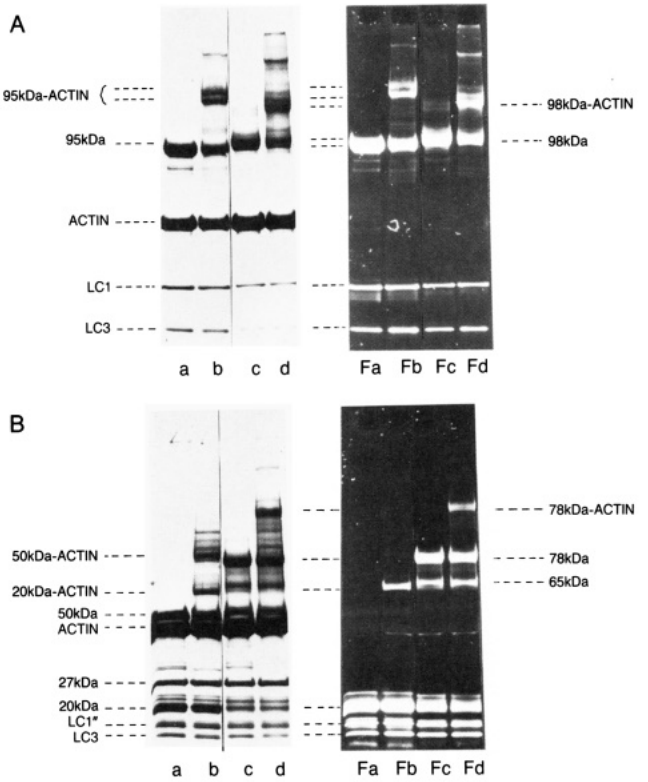

FIGURE 5: EDC-induced cross-linking between F-actin and (SH_2–Cys_{540})–pPDM–cross-linked S-1. (A) The reaction of fluorescent AEDANS–SH_1–S-1 (a, Fa) or AEDANS–SH_1–(SH_2–Cys_{540})–pPDM–S-1 (c, Fc) with EDC-activated F-actin was conducted as indicated under Materials and Methods; the protein samples were analyzed by polyacrylamide gel electrophoresis after 15 min of cross-linking (b, Fb and d, Fd, respectively). The gel was stained with Coomassie blue (a–d) or viewed under ultraviolet light (Fa–Fd). (B) F-Actin was cross-linked to trypsin-split AEDANS–SH_1–S-1 (a, Fa) and AEDANS–SH_1–(SH_2–Cys_{540})–pPDM–S-1 (c, Fc) as in (A). The gel pattern of the protein mixtures was analyzed after Coomassie blue staining (b and d, respectively) or under ultraviolet light (Fb and Fd, respectively).

of the 20-kDa fragment). Bridging was accomplished with either pPDM (12 Å long) or DTNB (2 Å long). The data of Figure 2 confirm that SH_2 was involved. CNBr cleavage of the cross-linked S-1 produces a 14.0-kDa fragment which is a covalent association of the P10 peptide (containing the fluorescent SH_1, SH_2, and an N-terminal glutamic acid residue) and the Cys-540-containing peptide (which pocesses a Gly at the N-terminus). Therefore, we can conclude that when pPDM reacts with SH_1-blocked S-1 it cross-links thiol SH_2 to Cys-540 (previously named SH_x) of the 50-kDa domain.

The 1.2-nm proximity (pPDM span) of residues 540 and 697 is relevant to the recent work of Ue (1987), who found a preexisting proximity of 0.8 nm (bimane span) between residues Cys-522 and Cys-707. The present result strengthens the idea proposed by Mornet et al. (1985a) that, in a topological sense, there exists a loop rendering contiguous the heavy-chain regions 522/540 and 697/707 as illustrated in Figure 6 (this should not be confused with β or Ω loops often studied in protein chemistry); in this loop is also located Lys-560, a site the proteolytic cleavage of which inhibits S-1 ATPase activity (Chaussepied et al., 1986b). The binding of nucleotide stabilizes the contacts between Cys-697 and Cys-540 by affecting the flexible heavy-chain conformation in this area. As is the case with the SH_1–SH_2 bridge (Wells & Yount, 1979, 1980), formation of this new intramolecular link between SH_2 and Cys-540 results in the entrapment of Mg nucleotide (Chaussepied et al., 1986a). This nucleotide

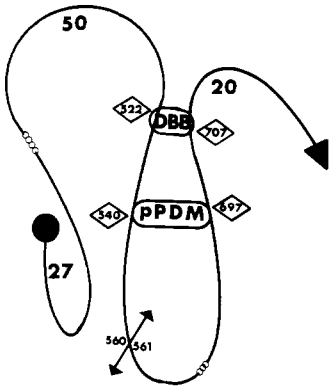

FIGURE 6: Schematic drawing of the S-1 heavy-chain "loop" structure illustrating the spatial proximity of the 522/540 and 697/707 regions as revealed by their intramolecular chemical cross-linking with dibromobimane (DBB) and *N,N'-p*-phenylenedimaleimide (pPDM). The arrow indicates the site of thrombin cleavage at Lys-560 which abolishes ATPase activity (Chaussepied et al., 1986b). The circles (O) denote the two protease-sensitive segments connecting the 27-, 50-, and 20-kDa fragments.

trapping event may not be due to the loss of SH_2 per se, because its modification does not induce extensive trapping (unpublished observation), but is rather due to a specific structural modification around this Cys-697 (SH_2). Various recent studies have related the direct or indirect nucleotide interaction to the region between residues 540 and 697 (Hozumi, 1983; Mornet et al., 1985b; Chaussepied et al., 1986c), strengthening the idea that the active site is not confined to the 27-kDa domain but also involves parts of the other domains.

The identification of Cys-540 as an anchor of the new bridge is important because we now have a new intramolecular distance helpful in S-1 modeling. Another interesting aspect was to identify the functional properties of this new stable S-1 derivative.

The CD spectral properties of S-1 in the near-ultraviolet are sensitive to its interaction with ATP and ADP (Murphy, 1974). This effect is seen near 258 nm (attributed to Phe) and 282 nm (attributed to Trp and Tyr; Burke et al., 1976). Compared to NEM-SH_1-S-1, SH_2-Cys_{540} bridge formation seems to affect the CD spectrum in a manner similar to that of ADP binding at the active site (Figure 2). In contrast, the effect of the SH_1-SH_2 cross-link on the S-1 CD spectrum seems related to the ATP effect.

Because in each case we used fresh preparations (less than 2 days old), we could calculate, on the basis of the known $t_{1/2}$ off rate of ADP, that both samples still contained 70–90% of the expected trapped nucleotide. In both cases, the trapped nucleotide is MgADP (Wells & Yount, 1980; Chaussepied et al., 1986a), so the different effect obtained with each cross-linked S-1 is mainly due to the different conformational constraints imposed by each intramolecular cross-link, and not due directly to the entrapped nucleotide.

The actin binding properties of the S-1 heavy chain also reflect S-1 conformations. As shown in Table I, in comparison with SH_1-SH_2 cross-linking which induces a 2000-fold decrease in the stability of the actin-S-1 interaction, SH_2-Cys_{540} cross-linking causes a decrease of only 10-fold in the stability. As previously reported, (SH_1-SH_2)-S-1 exhibits an affinity constant for actin similar to that obtained with the S-1-ADP·P_i complex (Chalovich et al., 1983; Katoh & Morita, 1984; Greene et al., 1986). On the contrary, (SH_2-Cys_{540})-S-1 attracts actin as strongly as does S-1-ADP. As recently described by Greene et al. (1986) for (SH_1-SH_2)-S-1, Mg nucleotide in solution cannot exchange with trapped ADP, but this reaction becomes possible when actin interacts with the cross-linked S-1. The actin interaction greatly changes the $t_{1/2}$ of the escape rate of trapped nucleotide in the new (SH_2-Cys_{540})-S-1 as well (data not shown).

Table II: Comparative States of (SH_2-Cys_{540})-S-1 and (SH_1-SH_2)-S-1 Containing Trapped Mg^{2+}-ADP

properties	(SH_2-Cys_{540})-S-1	(SH_1-SH_2)-S-1
$t_{1/2}$ off rate of Mg^{2+}-ADP[a] (days)	3–4	7–8
actin interaction		
affinity constant (M^{-1})	6.8×10^6	3.2×10^4
ionic strength effect	weakly sensitive	strongly sensitive
EDC cross-linking	unknown interface	acto-20 kDa, acto-50 kDa products
CD spectrum	M·ADP/M'·ADP	M*·ATP/M**ADP·P_i

[a] Determined at 4 °C in 50 mM Tris-HCl buffer, pH 8.0.

The actin interaction with the S-1–nucleotide complex is specifically influenced by salt concentration when ADP or ADP·P_i (ATP) is present in the active site (Highsmith, 1976; Chalovich et al., 1983; Arata, 1984; Katoh & Morita, 1984). We used this S-1 property further to characterize the new cross-linked S-1. It is clearly shown in Figure 4 that (SH_2-Cys_{540})-S-1 can be compared to the S-1–ADP complex, whereas (SH_1-SH_2)-S-1 is still related to the ADP·P_i (ATP) complex.

Finally, we studied the actin interface, using this new pPDM-cross-linked S-1, with EDC as the intramolecular cross-linker (Mornet et al., 1981; Sutoh, 1983). The actin cross-links to the native S-1 heavy chain, generating a complex migrating on the $NaDodSO_4$ gel as a doublet band of 175 and 185 kDa (corresponding respectively to actin cross-linked with the 20- and 50-kDa fragments). If MgATP or MgADP induces a specific change in the acto-S-1 relationship, the different nucleotides do not seem to affect the distribution of EDC cross-links between actin and the 20- and 50-kDa fragments (Chen et al., 1985). The (SH_1-SH_2)-S-1 derivative is also able to cross-link through its 20- and 50-kDa domains (unpublished observation; Chen et al., 1985). The cross-linked product we obtained with acto-(SH_2-Cys_{540})-S-1 complex is mainly composed of a single band of 150 kDa when the intact S-1 heavy chain is the starting material and 140 kDa with the 78-kDa derivative (20-kDa + 50-kDa fragments). Unfortunately, it was impossible to break the pPDM bridge without damaging the peptide bond, so we could not verify whether the actin interfaces of the 20- and 50-kDa fragments were present, partially absent, or totally changed in these new products.

Recently, Mornet et al. (1986), studying the interaction of actin with the bimane-cross-linked S-1 (bimane cross-links "SH_1" and "Cys_{522}"), showed that the cross-linked products migrating as 155 kDa (actin/S-1) and 125 kDa (actin/20 kDa–50 kDa) arose only from the covalent binding of actin and 50-kDa fragment. Although (SH_1-Cys_{522})-S-1 and (SH_2-Cys_{540})-S-1 certainly have some structural similarities, they have profound differences in their EDC-induced interaction with actin; for example, their actin cross-linked products display different masses.

Additionally, the 65-kDa (20 kDa–45 kDa) fragment does not cross-link with actin in contrast to the 20 and 45 kDa of un-cross-linked S-1 (Labbé et al., 1984). This last result suggests that the covalent union between (SH_2-Cys_{540})-S-1 and EDC–actin requires the structural integrity of the 50-kDa domain which is not the case for unmodified S-1.

Table II summarizes the properties of S-1 that has been cross-linked in two ways that both entrap MgADP. We

confirm that S-1 with the SH_1–SH_2 cross-link has a weak affinity for actin and a CD spectrum resembling that of S-1 that is hydrolyzing ATP, i.e., that is ligating "ADP·P_i" in a steady state. The S-1 with the Cys_{540}–SH_2 cross-link has a fairly strong affinity for actin (about one-tenth that of unmodified S-1) and a CD spectrum resembling that of S-1 equilibrated with MgADP. A great deal has been made of the analogy between the SH_1–SH_2 cross-link and M·ADP·P_i (Burke et al., 1976; Chalovich et al., 1983). With equal justification, we could analogize between the new cross-link and M·ADP (or M*·ADP), but both analogies are only tentative because in both cases MgADP is entrapped. While *differences* in properties between the two systems would logically be expected to result from structural differences caused by the different cross-links, there is no denying that the entrapped metal nucleotide complex may influence the properties themselves. Nevertheless, the grouping of the differences seems to us very suggestive. Two other contrasts in Table II would seem to refer to major differences in conformation—the tightness with which nucleotide is trapped and the sensitivity to ionic strength. The one result dissonant with the analogy between the effect of the Cys_{540}–SH_2 cross-link and that of binding MgADP seems to be the actin–EDC cross-linking which displays a different electrophoretic pattern than that obtained with the S-1–ADP complex. This discrepancy might be explained in at least two ways: (i) the pPDM-cross-linked S-1 migrates anomalously when cross-linked to actin but has the same actin interface as the S-1–ADP complex; (ii) the actin interface of (SH_2–Cys_{540})–S-1 is really different from that of the S-1–ADP species obtained in solution but is similar to some other S-1–ADP intermediate, e.g., the S-1′–ADP (Sleep & Hutton, 1980). Only a study of the actual structure of the actin-cross-linked products will answer this last point.

Finally, we note that the presence of a fluorescent label on SH_1 does not modify significantly most of the properties cited above (unpublished result). This makes the new (SH_2–Cys_{540})–S-1 derivative useful for different complementary studies either in solution in relation to the change occurring in the S-1 heavy-chain topography (Botts et al., 1984) or in the fiber (Burghardt & Ajtai, 1986; Ajtai & Burghart, 1987).

ACKNOWLEDGMENTS

We thank K. Ue for valuable help during the SH_x identification and A. Pantaloni for expert assistance in CD spectroscopy.

Registry No. pPDM, 3278-31-7; ADP, 58-64-0; ATP, 56-65-5; Cys, 52-90-4.

REFERENCES

Ajtai, K., & Burghardt, T. P. (1987) *Biochemistry 26*, 4517–4523.

Arata, T. (1984) *J. Biochem. (Tokyo) 96*, 337–347.

Botts, J., Muhlrad, A., Takashi, R., & Morales, M. F. (1982) *Biochemistry 21*, 6903–6905.

Botts, J., Takashi, R., Torgerson, P., Hozumi, T., Muhlrad, A., Mornet, D., & Morales, M. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2060–2064.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burghardt, T. P., & Ajtai, K. (1986) *Biochemistry 25*, 3469–3478.

Burke, M., Reisler, E., & Harrington, W. F. (1976) *Biochemistry 15*, 1920–1927.

Chalovich, J. M., & Eisenberg, E. (1982) *J. Biol. Chem. 257*, 2432–2437.

Chalovich, J. M., Greene, L. E., & Eisenberg, E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4909–4913.

Chaussepied, P., Mornet, D., & Kassab, R. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2037–2041.

Chaussepied, P., Mornet, D., Barman, E., Travers, F., & Kassab, R. (1986b) *Biochemistry 25*, 1141–1149.

Chaussepied, P., Mornet, D., & Kassab, R. (1986c) *Biochemistry 25*, 6426–6432.

Chen, T., Applegate, D., & Reisler, E. (1985) *Biochemistry 24*, 5620–5625.

Eisenberg, E., & Kielley, W. W. (1974) *J. Biol. Chem. 249*, 4742–4748.

Elzinga, M., & Collins, J. H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4281–4284.

Goodearl, A. J., Levine, B. A., & Trayer, J. P. (1985) *J. Muscle Res. Cell Motil. 6*, 71a.

Gray, W. R. (1972) *Methods Enzymol. 25*, 121–138.

Greene, L. E., Chalovich, J. M., & Eisenberg, E. (1986) *Biochemistry 25*, 704–709.

Hibberd, M. G., & Trentham, D. R. (1986) *Annu. Rev. Biophys. Chem. 15*, 119–161.

Highsmith, S. (1976) *J. Biol. Chem. 251*, 6170–6172.

Highsmith, S., & Eden, D. (1987) *Biochemistry 26*, 2747–2750.

Hozumi, T. (1983) *Biochemistry 22*, 799–804.

Hudson, E. N., & Weber, G. (1973) *Biochemistry 12*, 4154–4161.

Katoh, T., & Morita, F. (1984) *J. Biochem. (Tokyo) 96*, 1223–1230.

Kunz, P. A., Walzer, J. T., Watterson, J. G., & Schaub, M. C. (1977) *FEBS Lett. 83*, 137–140.

Labbe, J. P., Bertrand, R., Audemard, E., Kassab, R., Walzthony, D., & Walliman, T. (1984) *Eur. J. Biochem. 143*, 315–322.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lu, R. C., Moo, L., & Wong, A. G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 6392–6396.

Mornet, D., Pantel, P., Audemard, E., & Kassab, R. (1979) *Eur. J. Biochem. 100*, 421–431.

Mornet, D., Pantel, P., Bertrand, R., Audemard, E., & Kassab, R. (1980) *FEBS Lett. 117*, 183–188.

Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306.

Mornet, D., Ue, K., & Morales, M. F. (1985a) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1658–1662.

Mornet, D., Pantel, P., Audemard, E., Derancourt, J., & Kassab, R. (1985b) *J. Mol. Biol. 183*, 1658–1662.

Mornet, D., Ue, K., Chaussepied, P., & Morales, M. F. (1986) *Eur. J. Biochem. 159*, 555–561.

Murphy, A. J. (1974) *Arch. Biochem. Biophys. 163*, 290–296.

Offer, G., Moos, C., & Starr, R. (1973) *J. Mol. Biol. 74*, 653–679.

Rajasekharan, K., Sivaramakrishnan, M., & Burke, M. (1987) *Biophys. J. 51*, 317a.

Reisler, E., Burke, M., & Harrington, W. F. (1974) *Biochemistry 13*, 2014–2022.

Sleep, J. A., & Hutton, R. L. (1980) *Biochemistry 19*, 1276–1283.

Sutoh, K. (1983) *Biochemistry 22*, 1579–1585.

Takashi, R., Duke, J., Ue, K., & Morales, M. F. (1976) *Arch. Biochem. Biophys. 175*, 279–283.

Ue, K. (1987) *Biochemistry 26*, 1889–1894.

Wagner, P. D., & Weeds, A. G. (1977) *J. Mol. Biol. 109*, 455–473.

Wells, J. A., & Yount, R. G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4966–4970.
Wells, J. A., & Yount, R. G. (1980) *Biochemistry 19*, 1711–1717.
West, J. J., Nagy, B., & Gergely, J. (1967) *J. Biol. Chem. 242*, 1140–1145.
Wu, C. S. C., & Yang, J. T. (1976) *Biochemistry 15*, 3007–3014.

Kinetics of Nucleotide and Metal Ion Interaction with G-Actin†

Ewa Nowak,‡ Hanna Strzelecka-Golaszewska,‡ and Roger S. Goody*,§

Abteilung Biophysik, Max-Planck-Institut für medizinische Forschung, Jahnstrasse 29, D-6900 Heidelberg 1, Federal Republic of Germany, and Department of Muscle Biochemistry, Nencki Institute of Experimental Biology, PL-02-093 Warszawa, Poland

Received July 7, 1987; Revised Manuscript Received November 5, 1987

ABSTRACT: The kinetics of interaction of Ca^{2+} ions and nucleotides with G-actin have been investigated by making use of the enhancement of 1,N^6-ethenoadenosine 5′-triphosphate (ϵATP) fluorescence on binding to actin, the enhancement of 2-[[2-[bis(carboxymethyl)amino]-5-methylphenoxy]methyl]-6-methoxy-8-[bis(carboxymethyl)amino]quinoline (Quin-2) fluorescence on binding to Ca^{2+}, and the sensitivity of the fluorescence of an *N*-(iodoacetyl)-*N*′-(5-sulfo-1-naphthyl)ethylenediamine (1,5-AEDANS) group on Cys-374 to metal ion binding. It is concluded that metal ion dissociation is the rate-limiting step in nucleotide dissociation (0.016 s^{-1} for Ca^{2+} at pH 7.2 and 21 °C) and that earlier conclusions that metal ion release is relatively fast and subsequent nucleotide release slow are incorrect. Results presented here and obtained by others on the metal ion concentration dependence of the effective rate of nucleotide exchange can be interpreted in the light of this conclusion in terms of a limiting rate which corresponds to that of metal ion release and an "apparent" dissociation constant for Ca^{2+} which is without direct physical significance. This apparent dissociation constant is more than 2 orders of magnitude greater than the real dissociation constant of Ca^{2+} from the Ca–actin–ATP complex, which was estimated to be 2×10^{-9} M from a titration with Quin-2. Confirmation that the rate of Ca^{2+} release is rate limiting both in nucleotide dissociation reactions and in replacement of Ca^{2+} by Mg^{2+} was obtained with 1,5-AEDANS–actin, since both the replacement of Ca^{2+} by Mg^{2+} and the removal of Ca^{2+} to give the actin–ATP complex occurred at the same (slow) rate. Metal ion exchange kinetics (Mg^{2+}/Ca^{2+}) can be explained by use of the simplest possible kinetic model of interaction of both metal ions and with appropriate rate constants for the forward and reverse rate constants of metal ion binding. In particular, the rate of dissociation of Mg^{2+} is about 1 order of magnitude lower than that for Ca^{2+}. The data obtained, together with thermodynamic considerations, lead to the conclusion that the tightly bound Ca^{2+} cannot be bound as a complex with ATP. Kinetic evidence suggesting that a metal ion–nucleotide complex is recognized in the binding reaction at relatively high metal ion concentration thus requires that this is a further metal ion, possibly one of the "weakly" bound ones.

The kinetics and thermodynamics of nucleotide and metal ion interactions with G-actin have been the subjects of numerous investigations over the past 2–3 decades [e.g., Asakura (1961), Barany et al. (1962), Kuehl and Gergely (1969), Waechter and Engel (1975, 1977), Neidl and Engel (1979), Frieden et al. (1980), and Carlier et al. (1986)]. Several years ago, a consensus of opinion appeared to have been reached which may be summarized briefly as follows. ATP can bind to G-actin either in the presence or in the absence of divalent metal ions (normally Ca^{2+} or Mg^{2+}), but its affinity is much higher in their presence. Calcium binding to the so-called high-affinity site was considered to be a relatively rapid equilibrium reaction, but the rate constant for nucleotide release, which only occurs after dissociation of metal, was thought to be very low (ca. 0.02–0.05 s^{-1}) and to be rate limiting in nucleotide exchange or dissociation processes. The latter points, in particular the apparently relatively weak binding of Ca^{2+} even to the high-affinity site (ca. 10^5–10^6 M^{-1}) and the low rate constant for the release of nucleotide, appeared to be strongly supported by experiments on the rate of nucleotide exchange as a function of free Ca^{2+} (Kuehl & Gergely, 1969; Waechter & Engel, 1975). However, very recent evidence has been interpreted to indicate that Ca^{2+} binding is much stronger than previously thought and that its rate of dissociation from its complex with G-actin–ATP is much slower than that implied by earlier investigations (Gershman et al., 1986), although this latter point was not considered to be confirmed in most recent work (Carlier et al., 1986).

In this paper, evidence is presented which leads to a rationalization of the older and newer results. In the proposed scheme, which is consistent with all the data presented here and with relevant published data, Ca^{2+} release is slow, and subsequent release of ATP from its metal-free complex with G-actin is much faster.

Materials and Methods

Protein Preparations. Actin from rabbit skeletal muscle was obtained as described earlier (Drabikowski & Nowak,

† This work was supported by a fellowship from the Max-Planck-Gesellschaft zur Förderung der Wissenschaften (to H.S.-G.), by a fellowship from the Alexander von Humboldt Stiftung (to E.N.), and, in part, by the Polish Academy of Science within Project CPBP 1.04.

* Address correspondence to this author.
‡ Nencki Institute of Experimental Biology.
§ Max-Planck-Institut für medizinische Forschung.

0006-2960/88/0427-1785$01.50/0